Osteoarthritis and Cartilage

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Development of comprehensive functional genomic screens to identify novel mediators of osteoarthritis

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Summary

Objective: The aim of this study was to develop high-throughput assays for the analysis of major chondrocyte functions that are important in osteoarthritis (OA) pathogenesis and methods for high-level gene expression and analysis in primary human chondrocytes.

Methods: In the first approach, complementary DNA (cDNA) libraries were constructed from OA cartilage RNA and full-length clones were selected. These cDNAs were transferred into a retroviral vector using Gateway Technology. Full-length clones were over-expressed in human articular chondrocytes (HAC) by retroviral-mediated gene transfer. The induction of OA-associated markers, including aggrecanase-1 (Agg-1), matrix metalloproteinase-13 (MMP-13), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), collagen IIA and collagen X was measured by quantitative real-time polymerase chain reaction (QPCR). Induction of a marker gene was verified by independent isolation of 2−3 clones per gene, re-transfection followed by QPCR as well as nucleotide sequencing. In the second approach, whole cDNA libraries were transduced into chondrocytes and screened for chondrocyte cluster formation in three-dimensional agarose cultures.

Results: Using green fluorescent protein (eGFP) as a marker gene, it was shown that the retroviral method has a transduction efficiency of >90%. A total of 40 verified hits were identified in the QPCR screen. The first set of 19 hits coordinately induced iNOS, COX-2, Agg-1 and MMP-13. The most potent of these genes were the tyrosine kinases Axl and Tyro-3, receptor interacting kinase-2 (RIPK2), tumor necrosis factor receptor 1A (TNFR1A), fibroblast growth factor (FGF) and its receptor FGFR, MUS81 endonuclease and Sentrin/SUMO-specific protease 3. The second set of seven hits induced both Agg-1 and MMP-13 but none of the other markers. Five of these seven genes regulate the phosphoinositide-3-kinase pathway. The most potently induced OA marker was iNOS. This marker was induced 20−500 fold by seven genes. Collagen IIA was also induced by seven genes, the most potent being transforming growth factor β (TGFβ)-stimulated protein TSC22, vascular endothelial growth factor (VEGF) and splicing factor 3a. This screening assay did not identify inducers of collagen X.
The second chondrocyte cluster formation screen identified 14 verified hits. Most of the genes inducing cluster formation were kinases. Additional genes had not been previously known to regulate chondrocyte cluster formation or any other chondrocyte function.

Conclusions: The methods developed in this study can be applied to screen for genes capable of inducing an OA-like phenotype in chondrocytes on a genome-wide scale and identify novel mediators of OA pathogenesis. Thus, coordinated functional genomic approaches can be used to delineate key genes and pathways activated in complex human diseases such as OA.
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Key words: Chondrocytes, Cartilage, Osteoarthritis, Retrovirus.

Introduction

Osteoarthritis (OA) is the most prevalent joint disease. Most individuals over age 60 have radiographic or histological signs of OA and about half of them experience subjective symptoms[1]. Current therapeutic approaches are directed towards symptomatic relief because pharmacologic interventions that prevent disease progression are not available[2]. Many patients thus progress to advanced disease where total joint replacement surgery is indicated. The major pathological change and source of subjective symptoms and joint dysfunction is the progressive loss of articular cartilage. Other joint structures are also involved in the OA process. OA is associated with synovial inflammation, osteophyte formation and remodeling of the subchondral bone[3,4]. Although considered a degenerative disorder, the OA process is driven by activation of chondrocytes[5,6]. This cell activation is manifested by increased cell proliferation or cluster formation and cell death[7]. Chondrocytes produce a broad spectrum of extracellular matrix-degrading enzymes and inflammatory mediators including cytokines, prostaglandins, nitric oxide and other oxygen radicals. The formation of new tissue that is not hyaline articular cartilage but fibro cartilage also occurs. The presence of fibro cartilage and the expression of type X and IIA collagen are related to aberrant chondrocyte differentiation or de-differentiation[8,9]. These observations suggest that OA

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pathogenesis involves a complex series of gene expression changes leading to chondrocyte activation and release of inflammatory mediators. Previous approaches to develop OA therapies were primarily focused on extracellular matrix degradation[10−12]. Identification of new drug targets has recently been pursued by the analysis of gene expression patterns that are characteristic of OA. Several gene profiling studies demonstrate that a large number of genes are differentially expressed in OA vs normal cartilage and support the notion that OA is associated with chondrocyte activation and de-differentiation[13,14]. The full potential of gene profiling studies can only be realized if efficient and biologically meaningful assays for the analysis of function of differentially expressed genes are available. Gene profiling is also limited by discrepancies between mRNA, protein levels and protein function and by not being able to detect small differences in gene expression levels.

The objectives of the present study were to develop high-throughput assays for the analysis of major chondrocyte functions that are important in OA pathogenesis and to develop methods for high-level gene expression and analysis in primary human chondrocytes. This will enable us to exploit sequence information and gene expression profiling data in the search for novel OA therapeutics on a genome-wide scale.

Materials and methods

CARTILAGE PROCUREMENT AND PROCESSING

Cartilage from the femoral condyles and tibial plateaus of human knee joints was obtained at autopsy from donors without known history of joint disease or from healthy organ donors. Tissue was also obtained at the time of total joint replacement surgery from patients with OA. All samples were graded according to a modified Mankin scale[15].

CHONDROCYTE ISOLATION AND CULTURE

Cartilage samples were rinsed in phosphate buffered saline (PBS), minced and digested with protease from *Streptomyces griseus* (Sigma, St. Louis, MO) and collagenase-2 (Worthington Biochemicals, Lakewood, NJ). Chondrocytes were seeded at high density (1×10^6 cells/well) in 6-well plates in high glucose Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated bovine serum and maintained in a CO_2 incubator (5%, 37°C) until cells reached 80% confluence.

RNA ISOLATION FROM CARTILAGE

RNA was isolated from flash frozen cartilage by homogenizing the tissue in a freezer mill and extracting the homogenate in Trizol (Life Technologies, Rockville, MD). The samples were extracted with chloroform, centrifuged at $15,000 \times g$ for 20 min and the aqueous phase was collected. An equal volume of 70% ethanol was added, mixed and applied to RNeasy columns (Qiagen, Valencia, CA). RNA concentrations were determined using Ribo-Green reagent (Molecular Probes, Eugene, OR).

SELECTION OF COMPLEMENTARY DNA (cDNA) CLONES FOR HIGH-THROUGHPUT SCREENING

cDNA libraries were constructed from OA cartilage RNA using the Superscript Choice System® (Invitrogen, Carlsbad, CA) in pCMVSport6 vector as per manufacturer's protocols. Approximately 50,000 clones from these cDNA libraries were arrayed into 96-well plates and sequenced at the 5′ end. The raw sequences were pre-processed[16,17] to remove low quality and vector sequences. Clones which had a minimum sequence of at least 150 bp were blasted against the human Refseq database for annotation[18]. Annotations were assigned if the identity was greater than 95% in the 150 bp region tested. Clones likely to encode full-length proteins were selected based on the presence of the initiation codon in the 5′ end sequence. A subset of the full-length clones likely to have roles in signal transduction were retrieved using QBOT (Genetix, New Milton, UK).

PREPARATION OF PLASMID DNA FROM FULL-LENGTH cDNA CLONES

The selected cDNA clones were grown in 96-deep-well blocks (Qiagen) containing 1.0 ml of Terrific broth (Sigma) and ampicillin (40 μg/ml). Plasmid DNA was isolated using Biorobot 8000 (Qiagen) using manufacturer's protocols.

GATEWAY TRANSFER OF FULL-LENGTH cDNA CLONES

The Gateway technology (Invitrogen) uses the lambda recombination system to facilitate transfer of heterologous DNA sequences (flanked by modified att sites) between vectors. Two recombination reactions constitute the basis of the Gateway technology. BP reaction facilitates recombination of an attB site with an attP site to create an intermediate attL site. This reaction is catalyzed by BP clonase enzyme mix. LR reaction facilitates recombination of an attL substrate with an attR substrate (destination vector) to create an attB containing retroviral expression vector. This reaction is catalyzed by LR clonase enzyme. BP and LR recombination reactions were carried out according to the one-tube protocol as described by the manufacturer (Invitrogen) but were miniaturized to a 96-well format. The retroviral vector used in this study was built in-house and is similar to Retro-X™ Q Vectors (BD Biosciences Clontech, Palo Alto, CA). The reaction products were transformed into DH5α cells (Invitrogen) and the final transformation mixture was added to each well of duplicate 48-deep-well block containing LB agar/40 μg/ml zeocin (Invitrogen) and grown at 37°C overnight. Single colonies from each well were inoculated to 1 ml Terrific broth/zeocin (40 μg/ml) and grown overnight at 37°C with shaking at 300 rpm. Plasmid DNA was isolated using the Biorobot 8000 (Qiagen).

PRODUCTION OF RETROVIRAL SUPERNATANTS

GP2-293 packaging cells (BD Biosciences Clontech) were seeded (5×10^4 cells per well) in 96-well Ploy-D-Lysine (PDL) plates (BD Biosciences) 16−24 h prior to transfection in antibiotic-free DMEM containing 10% fetal bovine serum (FBS, Invitrogen). Gateway® retroviral constructs (150 ng) and envelope vector pVPack-VSV-G (150 ng; Stratagene, La Jolla, CA) were co-transfected into packaging cells using Lipofectamine 2000 reagent (Invitrogen). The transfection media was replaced with complete media containing antibiotics 16−24 h after transfection and the viral supernatant was collected after 48 h.

RETROVIRAL TRANSDUCTION OF PRIMARY CHONDROCYTES

Primary chondrocytes (passage 3 or less) were seeded at 1×10^4 cells/well in 96-well plates, 24 h prior to transduction

at which time the media was replaced with 100 μl viral supernatant and 100 μl complete media supplemented with 20 mM (N-[2-Hydroxyethyl] piperazine-N' -[2-ethane-sulfonic acid]) (HEPES) and 16 μg/ml polybrene. Cells were centrifuged in a swinging bucket rotor at 32°C, 1000 × *g*, for 1.5 h[19]. The media was replaced after 16−24 h with fresh media and cells were incubated for an additional 48 h.

RNA ISOLATION AND QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (QPCR)

Total RNA was isolated from retrovirally transduced chondrocytes in 96-well plates using the BioRobot 8000 (Qiagen) and Qiagen RNeasy 96 Biorobot reagents. The optional on-column DNase I digestion was employed to eliminate contaminating genomic DNA. First strand cDNA was synthesized using random primers with the High-Capacity cDNA Archive kit (PE Applied Biosystems, Foster City, CA) in a 100 μl reaction volume.

Real-time PCR was performed in a 384-well format on the ABI Prism 7900HT Sequence Detection System (PE Applied Biosystems) using SYBR Green PCR Master Mix (PE Applied Biosystems). The cDNA template and PCR mix were distributed using the Biomek FX liquid handling robot (Beckman Coulter, Fullerton, CA). A 20 μl reaction contained 5 μl cDNA, 50 nM forward and reverse primers, and SYBR Green PCR Master Mix. The default cycling program was followed by a dissociation stage whereby a melting curve was generated to confirm the specificity of the PCR product and the absence of primer-dimer formation. The following primers were used: Aggrecanase-1 forward 5′-TTTCCCTGGCAAGGACTATGA-3′; Aggrecanase-1 reverse 5′-AATGGCGTGAGTCGGGC-3′; matrix metalloproteinase-13 (MMP-13) forward 5′-TGATCTCTTTTGGAAT TAAGGAGCAT-3′; MMP-13 reverse 5′-ATGGGCATCTC CTCCATAATTTG-3′; cyclooxygenase-2 (COX-2) forward 5′-AAATTGCTGGCAGGGTTGC-3′; COX-2 reverse 5′-TTTCTGTACTGCGGGTGGAAC-3′; inducible nitric oxide synthase (iNOS) forward 5′-GCAAACCTTCAAGGCAGCC-3′; iNOS reverse 5′-TGCTGTTTGCCTCGGACAT-3′; Collagen Type IIa forward 5′-ACGCTGCTCGTCGCCG-3′; Collagen Type IIa reverse 5′-GCCAGCCTCCTGGACAT CCT-3′; Collagen Type X forward 5′-ACCCAACAC CAA GACACAGTTCT-3′; Collagen Type X reverse 5′-TCTT ACTGCTATACCTTTACTCTTTATGGTGTA-3′; glyceralde-hyde-3-phosphate dehydrogenase (GAPDH) forward 5′-ATGGGGAAGGTGAAGGTCG-3′; GAPDH reverse 5′-TAAAAGCAGCCCTGGTGACC-3′. Changes in gene expression, normalized to GAPDH, relative to cells transfected with the retroviral vector containing no cDNA insert were calculated using the Comparative Ct method, as outlined in ABI User Bulletin #2 (PE Applied Biosystems).

CONSTRUCTION OF OA CARTILAGE cDNA LIBRARIES IN RETROVIRAL VECTORS

One microgram of polyA$^+$ RNA was isolated from 200 μg of total RNA using the Dynabead mRNA purification kit (Dynal, Lake Success, NY). The library was constructed using the Superscript Choice System in the pENTR 2.0 vector (Invitrogen). The Gateway transfer system was used to transfer the cDNA libraries into the retroviral vector, as described above. The libraries were amplified on selective solid medium. PCR of library DNA before and after the transfer showed a similar representation of high, medium and low-abundant cDNAs (data not shown).

PRODUCTION OF VIRAL SUPERNATANTS AND TRANSDUCTION OF CHONDROCYTES

The procedures used were as described above except that DNA from a whole cDNA library was used instead of individual clones. One microgram of OA cDNA library DNA and 1 μg of pVpack-VSVG plasmid were used to produce viral supernatants. The viral supernatants were spinfected into human articular chondrocytes (HAC, Cell Applications, San Diego, CA) in 6-well plates.

ASSAY FOR CHONDROCYTE CLUSTER FORMATION

Three days after retroviral transduction, cells were trypsinized and suspended in 0.4% low melt agarose (Life Technologies, Rockville, MD) in complete DMEM (Invitrogen) at a density of 1×10^4 cells/ml. Cells (8 ml) were pipetted into tissue culture plates (10-cm) containing 0.7% agarose in DMEM medium containing 10% FBS (Invitrogen). The agarose was allowed to solidify at room temperature and the agarose-chondrocyte cultures were incubated at 37°C. Transduced chondrocytes were assayed in agarose cultures for 3−4 weeks for cluster formation. Chondrocyte response was based on the number of clones formed that were greater than 50 μm in diameter. Clones were visualized using an Olympus IX70 inverted microscope (Olympus America, Inc., Melville, NY) with a 4× objective under bright field illumination. Each culture dish was photographed at five different microscope fields on three replicate plates and digitally captured on an Olympus MagnaFire CCD camera and software (Olympus). Each image was then analyzed using Image-Pro Plus version 4.5 software from Media Cybernetics Inc (Media Cybernetics, Silver Spring, MD). Each assay included basic fibroblast growth factor (bFGF)-transduced cells as a positive control and an empty vector plate as the negative control. Transgenes were rescued by PCR and identified by sequencing.

RESCUE OF CLONES BY PCR AND IDENTIFICATION BY NUCLEOTIDE SEQUENCING

Clones greater than 50 μm in size were identified using an Olympus 1X70 inverted microscope under 20× magnification and picked using a hand pipettor and seeded directly into 6-well cluster plates (BD Biosciences Clontech) at one clone per well. Clones were allowed to expand in monolayer culture (DMEM, 10% FBS) until confluent. RNA was isolated using RNeasy 96 (Qiagen). Real-Time Polymerase Chain Reaction (RT-PCR) was performed using 96-well format (Clontech Advantage RT-PCR kit using Perkin Elmer Amplitaq Gold) and primers for the AttB sites: AttB1 5′-CAAGTTTGTACAAAAAAGC-3′ and AttB2 5′-ACCACTTTGTACAAGAAAG-3′ (Gateway System, Invitrogen). The rescued cDNA was subcloned using the TOPO-TA cloning kit (Invitrogen) and identified by nucleotide sequencing.

DNA MICROARRAY ANALYSIS

Total RNA was prepared from human cartilage knee tissues using Qiagen RNeasy kit (Qiagen, Valencia, CA). Double stranded DNA was synthesized from 5 μg of total RNA using a primer containing poly(dT) and T7 polymerase promoter sequence. *In vitro* transcription with the double-stranded DNA as a template in the presence of biotinylated Uridine triphosphate (UTP) was performed using the protocol provided by Affymetrix (Santa Clara, CA). Biotinylated complementary RNA (cRNA) was purified, fragmented and

hybridized to U95A arrays (Affymetrix) following manufacturer's manual. The hybridized arrays were then washed and stained with streptavidin-phycoerythrin and scanned with a Hewlett Packard GeneArray Scanner. The average difference was calculated using the Affymetrix software version 4 using a target intensity of 1500. Results are shown as average expression level, which is the arithmetic mean of the average difference measured for human knee cartilage RNA from 28 patients profiled on U95A Affymetrix gene chips. Values less than 150 are not significantly different from noise.

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Cartilage was fixed with 4% paraformaldehyde and embedded in paraffin to cut 5 μm sections. The tissue sections were placed on slides, deparaffinized in toluene, and dehydrated in graded series of ethanol, then washed in PBS and in 0.2% peroxidase. The presence of Tyro-3 and its ligand Gas-6 was determined using the avidin-peroxidase method using antibodies to human Tyro-3 and human Gas-6 (R&D Systems, Minneapolis, MN).

Results

EFFICIENCY OF RETROVIRAL TRANSDUCTION OF CHONDROCYTES

A retroviral gene transfer method was developed for HAC. Using green fluorescent protein (eGFP) as a marker gene, it was shown that >70% of the chondrocytes expressed the transgene [Fig. 1(A)]. Development of the next series of retroviral vectors which included a puromycin selectable marker increased the transfection efficiency to >90% (data not shown).

To demonstrate that retroviral gene transfer is effective in activating HAC, platelet-derived growth factor (PDGF), a known inducer of aggrecanase-1 (Agg-1) and MMP-13 in chondrocytes was expressed[20]. PDGF gene expression in chondrocytes resulted in significant increases in both Agg-1 and MMP-13 mRNA levels as measured by QPCR [Fig. 1(B)]. This data suggests that retroviral-mediated gene transfer can be used to efficiently introduce genes into HAC and results in the induction of the expected biological response.
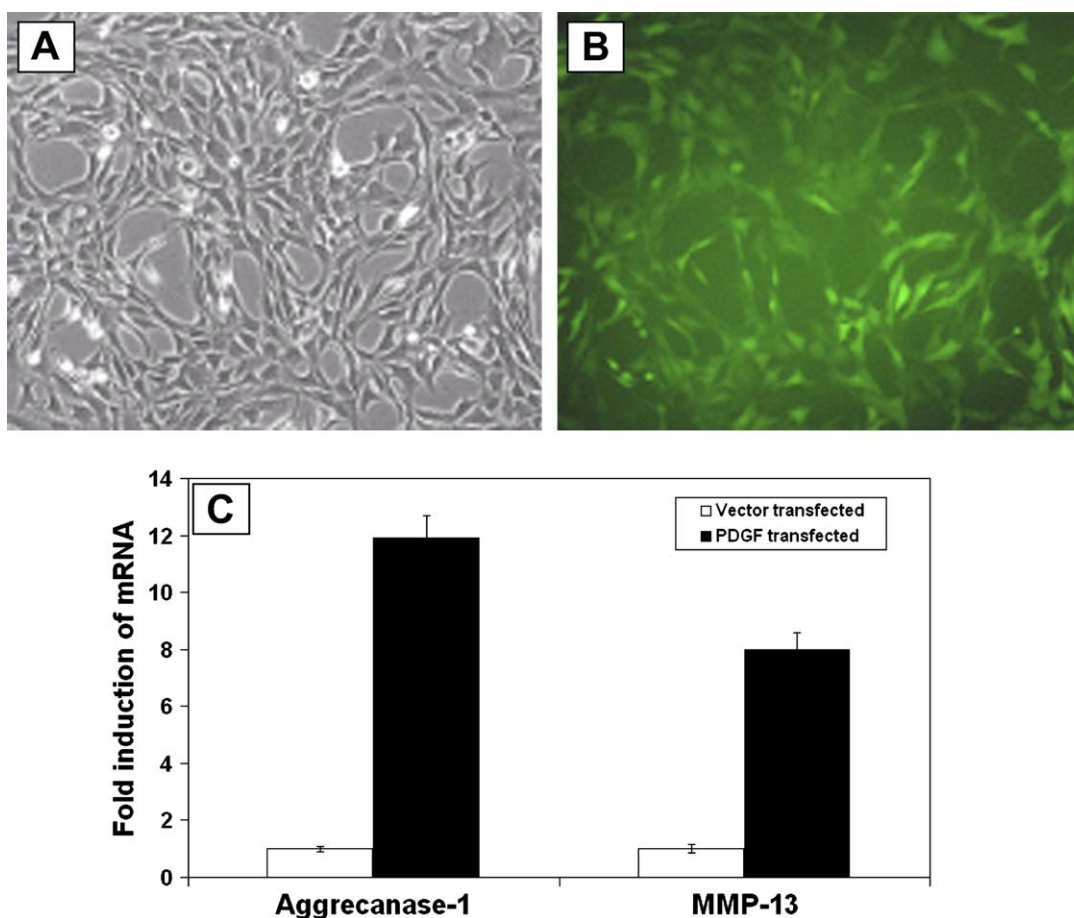

Fig. 1. Quantitation of retroviral-mediated gene transfer efficiency using eGFP and validation of the real-time PCR screen using PDGF as a test gene in HAC. HAC were transfected with eGFP reporter plasmid by retroviral-mediated gene transfer as described in the Methods section. eGFP-expressing cells were visualized using an inverted fluorescent microscope (Olympus, 1X70). (A) Represents bright light image and (B) represents fluorescent image. FACS analysis of transfected cells suggests >70% transduction efficiency. Using the same method, a PDGF cDNA clone was over-expressed in chondrocytes. RNA was harvested 72 h post-transduction and changes in expression of MMP-13 and Agg-1 mRNA were detected by QPCR (C).
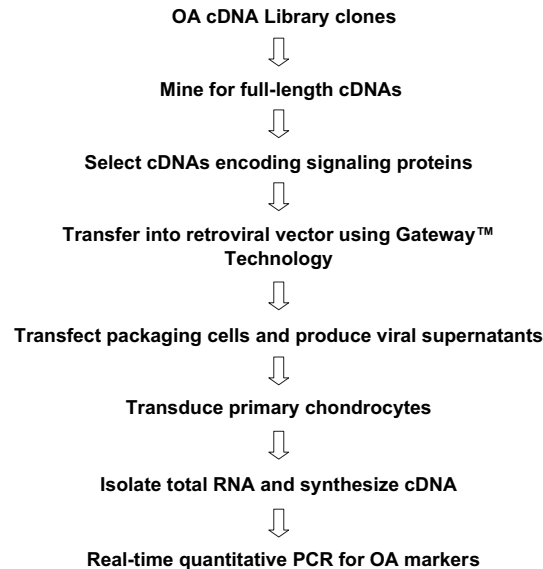
OA cDNA Library clones

⇩

Mine for full-length cDNAs

⇩

Select cDNAs encoding signaling proteins

⇩

Transfer into retroviral vector using Gateway™ Technology

⇩

Transfect packaging cells and produce viral supernatants

⇩

Transduce primary chondrocytes

⇩

Isolate total RNA and synthesize cDNA

⇩

Real-time quantitative PCR for OA markers

Fig. 2. Flow chart of high-throughput real-time PCR screen. The screen was run using 1200 cDNAs as described in the Methods section. Primers for the markers were designed with Primer Express software (PE Applied Biosystems) under default parameters and reaction conditions. Changes in gene expression of OA markers were quantitated as follows: Amplification of GAPDH was used to normalize the amount of cDNA added to the reaction. Changes in gene expression, normalized to GAPDH, relative to cells transfected with the retroviral vector containing no cDNA insert were calculated using the Comparative Ct method according to the formula $2^{-\Delta\Delta CT}$, as outlined in ABI User Bulletin #2 (PE Applied Biosystems).

SCREENING OF cDNAs FROM OA LIBRARIES FOR INDUCTION OF OA-LIKE GENE EXPRESSION PATTERNS IN CHONDROCYTES

An overview of the screen is shown in Fig. 2. cDNA libraries were constructed with RNA isolated from human OA cartilage and sequenced. A set of 1200 full-length cDNAs that encode proteins implicated to have a role in signal transduction such as kinases, receptors, transcription factors, enzymes, or secreted factors were selected. The clones were transferred from the pCMVSport6 vector to a retroviral vector. Retroviral plasmids were used to produce high-titer viral stocks by transient transfection of the GP2-293 packaging cell line. The 1200 full-length cDNAs were individually expressed in HAC by transducing cells with the viral supernatants. Total RNA was isolated 72 h post-infection and used to produce cDNA. QPCR was used to identify inducers of OA phenotypic markers: de-differentiation/hypertrophy (collagen type IIA and collagen type X), inflammation (iNOS and COX-2) and matrix degradation (Agg-1 and MMP-13).

The hits were verified by independent isolation of 2–3 clones per gene, re-transfection followed by QPCR as well as nucleotide sequencing. A total of 40 verified hits were identified in this screen (Table I). Hits are defined as genes that induced mRNA expression of at least one OA phenotypic marker by three fold or more. The hits in Table I are grouped according to the number of OA markers they induced. The first set of 19 hits coordinately induced the four markers iNOS, COX-2, Agg-1 and MMP-13. The most potent of these genes were the two related tyrosine kinases Tyro-3 and Axl, receptor interacting kinase-2 (RIPK2), tumor necrosis factor receptor 1A (TNFR1A), fibroblast growth factor (FGF) and its receptor FGFR, the MUS81 endonuclease and Sentrin/SUMO-specific protease 3. The second set of seven hits induced both Agg-1 and MMP-13 but none of the other markers. Five of these seven genes regulate the

PI3-kinase pathway. Genes that induced only one OA marker either increased iNOS or collagen IIA expression. It is of interest that none of the genes that induced collagen IIA induced any of the other markers. The most potently induced OA marker was iNOS. This marker was induced by seven genes by 20–500 fold. Collagen IIA was induced by seven genes, the most potent being transforming growth factor β (TGFβ)-stimulated protein TSC22, vascular endothelial growth factor (VEGF) and splicing factor 3a. This screening assay did not identify any inducers of collagen X.

IDENTIFICATION OF GENES THAT INDUCE CHONDROCYTE CLONING OR CLUSTER FORMATION

OA cartilage also features chondrocyte proliferation or cloning which leads to formation of characteristic chondrocyte clusters[21]. In three-dimensional cultures, growth factors and other stimuli induce chondrocyte cluster formation[22,23]. To first validate this experimental approach we demonstrated that chondrocyte cluster formation could be induced by retroviral transfer of a single growth factor gene. Retroviral transfer of bFGF induced chondrocyte cluster formation to a similar extent as recombinant bFGF protein (Fig. 3). A functional screen was then developed to identify inducers of chondrocyte cluster formation on a genome-wide scale (Fig. 4). Chondrocytes were retrovirally transduced with an OA cDNA library and transferred to three-dimensional agarose cultures. Large clusters were isolated after 3–4 weeks, transgenes were rescued by PCR and identified by sequencing. All hits identified using this approach were verified by determining their cluster forming potential in separate gene transfer experiments. Table II lists 14 verified hits. The first seven hits were identified in the chondrocyte cloning screen. The rest were identified in the QPCR screen and also found to be positive in the chondrocyte cloning screen. Identification of bFGF which is

Table I

High-throughput real-time PCR screen identifies novel mediators of OA phenotype. List of verified hits obtained from the HT-real-time PCR screen which induced at least one marker of OA > 3 fold are shown. The hits are divided into groups based on the number of markers they induced. The table also shows the expression levels of the hits in normal human cartilage based on Affymetrix DNA array analysis. Results are shown as average expression level, which is the arithmetic mean of the averages measured for human knee cartilage RNA from 28 donors profiled on U95A Affymetrix gene chips. Values less than 150 are not significantly different from the noise. NP: not present on U95A chip

Accession number	Average expression level in cartilage	Gene description	Fold increase in mRNA				
			Agg-1	MMP-13	iNOS	COX-2	Collagen IIA
Inducers of four OA markers							
NM_006293	570	TYRO-3 protein tyrosine kinase	51	29	400	15	
NM_021913	430	AXL receptor tyrosine kinase transcript variant 1	23	19	42	10	
NM_025128	201	MUS81 endonuclease	32	40	9	4	
NM_001065	1431	Tumor necrosis factor receptor superfamily, member 1A	28	20	205	6	
NM_003821	182	Receptor-interacting serine-threonine kinase 2	22	40	43	11	
NM_023107	2100	Fibroblast growth factor receptor 1	18	18	39	3	
NM_015670	329	Sentrin/SUMO-specific protease 3	15	7	11	4	
NM_015318	239	Rho-specific guanine nucleotide exchange factor p114	14	9	9	5	
NM_005198	196	Choline kinase like	10	24	40	2	
NM_000800	556	Fibroblast growth factor 1 (acidic) transcript variant 1	10	21	48	3	
NM_024689	NP	Hypothetical protein (FLJ14103)	9	5	3	5	
NM_021230	NP	Myeloid/lymphoid leukemia 3	8.6	3.7	4.8	2.3	
NM_003952	318	Ribosomal protein S6 kinase 70 kDa, polypeptide 2	7	15	48	2	
NM_002419	362	Mitogen-activated protein kinase kinase kinase 11	7	5	7	3	
NM_003656	282	Calcium/calmodulin-dependent protein kinase I	7	4	5	3	
NM_004039	7750	Annexin A2	6	16	30	2	
NM_203371	NP	Novel gene abundant in late OA	5	6	4	6	
NM_032411	NP	Esophageal cancer related gene 4 protein (ECRG4)	5	2	7	24	
NM_172171	462	Calcium/calmodulin-dependent protein kinase 2 gamma transcript variant 1	4	2	14	6	
Inducers of two OA markers							
NM_000294	282	Phosphorylase kinase, gamma 2 (testis)	12	31			
NM_002037	200	FYN oncogene related to SRC, FGR, YES	8	6			
NM_005163	215	v-akt murine thymoma viral oncogene homolog 1	8	9			
NM_002649	383	Phosphoinositide-3-kinase, catalytic, gamma polypeptide	6	4			
NM_174574	NP	Phosphoinositide-3-kinase, catalytic, alpha polypeptide	5	3.5			
NM_019884	672	Glycogen synthase kinase 3 alpha	5	9			
NM_006169	7250	Nicotinamide *N*-methyl transferase	4	12			
Inducers of one OA marker							
NM_005011	386	Nuclear respiratory factor 1			528		
NM_001663	1293	ADP-ribosylation factor 6			150		
BC033864	580	Similar to branched chain amino transferase 1			100		
NM_001945	237	Diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor)			33		
NM_000966	355	Retinoic acid receptor, gamma			25		
NM_000401	198	Exostoses (multiple) 2			23		
BC045575	NP	Similar to Adenosine diphosphate (ADP)-ribosylation factor 1			19		
NM_006022	4372	Transforming growth factor beta-stimulated protein TSC22					55
NM_003376	5270	Vascular endothelial growth factor					32
NM_006802	294	Splicing factor 3a, subunit 3					23
NM_018725	278	Interleukin 17B receptor					14
NM_001895	152	Casein kinase 2, alpha 1 polypeptide					13
NM_152912	NP	Mitochondrial translational initiation factor 3					12
NM_021975	1900	v-rel reticuloendotheliosis viral oncogene homolog A, p65 (avian)					12

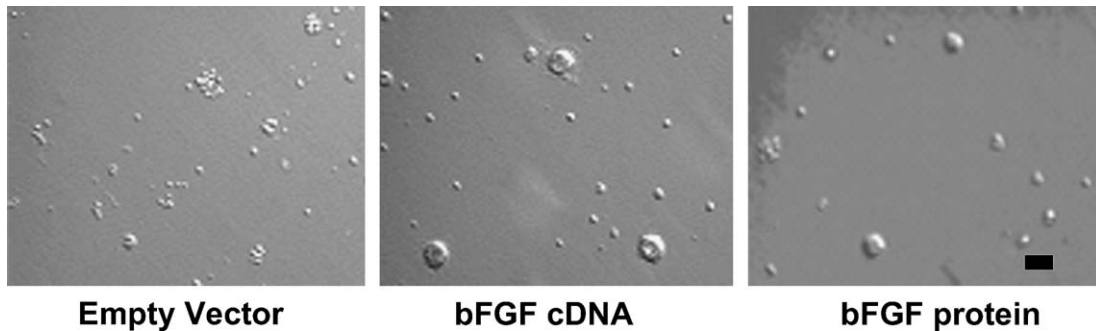**Empty Vector**   **bFGF cDNA**   **bFGF protein**

Fig. 3. Chondrocyte cloning induced by bFGF cDNA and bFGF protein. Chondrocytes were retrovirally transduced with bFGF or with empty vector and maintained in agarose cultures for 4 weeks. Non-transduced cells were stimulated with bFGF (100 ng/ml). The figure shows colonies >50 μm after 4 weeks in culture. First image represents chondrocytes transduced with empty vector; second image represents chondrocytes transduced with bFGF cDNA clone; third image represents normal chondrocytes that were cultured in the presence of bFGF protein (100 ng/ml) added exogenously.

a known inducer of chondrocyte cluster formation as a hit further validates the experimental approach. The other hits such as IL-17 receptor were previously known to promote cartilage degradation and joint inflammation[24,25] but not chondrocyte proliferation. C1r, a component of the classical complement pathway has been known to be produced by chondrocytes[26] but this is the first indication that it can regulate chondrocyte function. Most of the inducers of cluster formation are kinases, including PI3K, calcium calmodulin dependent protein kinase 1, choline kinase and casein kinase 2. Complex Oligomeric Matrix Protein (COMP) is a biochemical marker of cartilage degradation

Transfer OA cDNA libraries into retroviral vector using Gateway™ Technology

⇩

Transfect packaging cells and produce viral supernatants

⇩

Transduce primary chondrocytes

⇩

Plate transduced cells in agarose and incubate 3-4 weeks

⇩

Pick chondrocyte clusters larger than 50 μm in size and expand in monolayer

⇩

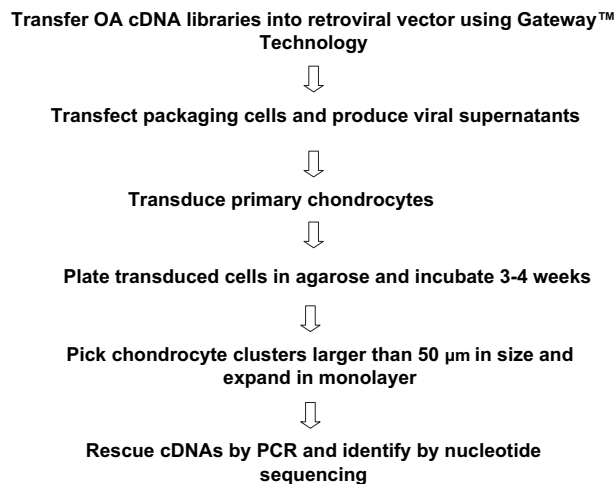**Rescue cDNAs by PCR and identify by nucleotide sequencing**

Fig. 4. Flow chart of high-throughput screen for chondrocyte cloning. An OA cDNA library was separately transferred into retroviral vectors using Gateway technology, packaged, and used to transduce HAC. Transduced chondrocytes were assayed in agarose cultures for 3−4 weeks for cluster formation. Clones were observed using an Olympus IX70 inverted microscope (Olympus America, Inc., Melville, NY) with a 4× objective under bright field illumination. Each culture dish was photographed at five different microscope fields on three replicate plates and digitally captured on an Olympus MagnaFire CCD camera and software (Olympus). Each image was then analyzed using Image-Pro Plus version 4.5 software from Media Cybernetics Inc (Media Cybernetics, Silver Spring, MD). Each assay included basic FGF transduced cells as a positive control, and an empty vector plate as the negative control. Clusters >50 μm in diameter were collected. Transgenes were recovered by PCR and identified by nucleotide sequencing.

and its function is related to the binding of collagen[27,28] and has been for the first time shown to have a role in chondrocyte cloning. Fig. 5 shows distribution of the hits by protein classes. Majority of the hits were kinases followed by others, enzymes, receptors and secreted factors.

EXPRESSION OF HITS IN HUMAN ARTICULAR CARTILAGE

To provide a basis for the function of the genes identified in the screens, we confirmed expression in articular cartilage. Results from DNA microarrays showed that all the hits that were represented on the array are expressed in cartilage (Tables I and II). For Tyro-3, the most potent inducer of OA markers, and its ligand Gas-6[29] we also performed immunohistochemistry analysis of human articular cartilage. Tyro-3 and Gas-6 showed increased expression in OA cartilage in chondrocyte clusters and in the mid and deep zones (Fig. 6).

Discussion

Genomic approaches to advance insight into the pathogenesis of OA and to identify targets for disease modifying therapy have relied on gene expression profiling[13,14], and the search for mutations in OA-affected families and animal strains[30−32]. Gene expression profiling has been successful in the identification of genes that are differentially expressed in OA-affected cartilage, some of which are new targets for drug development. The search for mutations that predispose to the development of OA has been successful in identifying mutations in extracellular matrix genes that account for a small subset of all OA patients. The clinical diversity of OA is likely to result from complex interactions of numerous gene products that have yet to be identified[32].

The objective of the present study was to develop and test novel genomic approaches to identify regulators of OA pathogenesis on a genome-wide basis. This involved the development or adaptation of three principal components.

The first component was to obtain full-length clones of genes that are expressed in OA cartilage. The cDNA libraries that were prepared from OA cartilage RNA contained approximately 5000 full-length clones, a number sufficient to establish feasibility of a large-scale genetic screening assay. cDNAs encoding proteins that have a role in signal transduction (1200 cDNAs) were selected to develop high-throughput functional screens.

Table II

Chondrocyte cloning screen reveals novel inducers of chondrocyte cluster formation. All genes listed induced significantly (Student's t test) more clusters (>50 µm diameter) than the empty vector. Values represent number of clusters >50 µm diameter as a percentage of the number induced by bFGF. Gene expression levels in cartilage were determined and expressed as explained in Table I. NP: not present on U95A chip

Accession number	Average expression level in cartilage	Gene description	No. of clusters (% bFGF)
NM_004207	1552	*Homo sapiens* solute carrier family 16 (monocarboxylic acid transporter	59.4
NM_001733	1694	C1r complement component	22.5
NM_032732	NP	IL-17 receptor-like (IL-17RC)	70.2
NM_021074	790	NADH dehydrogenase (ubiquinone) flavoprotein 2 (24 kDa) (NDUFV2)	92.5
NM_032548	NP	Ankyrin repeat and BTB/POZ domains (BPOZ)	21.8
NM_000095	10,249	Cartilage oligomeric matrix protein	55.3
NM_002006	586	Basic fibroblast growth factor	100.0
NM_002649	383	PI3-kinase, catalytic, gamma polypeptide	149.3
NM_003656	282	Calcium calmodulin dependent protein kinase 1	117.3
NM_005198	196	Choline kinase like	94.6
NM_021230	NP	Myeloid/lymphoid leukemia 3	125.1
NM_015318	239	Rho-specific guanine nucleotide exchange factor p114	54.9
NM_003376	5270	Vascular endothelial growth factor	60.3
NM_001895	152	Casein kinase 2, alpha 1 polypeptide	48.0

The second component was to develop an efficient gene expression system for chondrocytes. We first established a method to rapidly transfer cDNAs from pCMVSport6 vector into retroviral vectors using Gateway technology. Retroviral gene transfer was subsequently adapted to chondrocytes and found to be highly efficient, resulting in transduction of the majority of cells.

The third component was to develop high-throughput assays for chondrocyte functions that are relevant to OA pathogenesis. The selected chondrocyte functions included extracellular matrix degradation, inflammation and chondrocyte de-differentiation. As mediators of extracellular
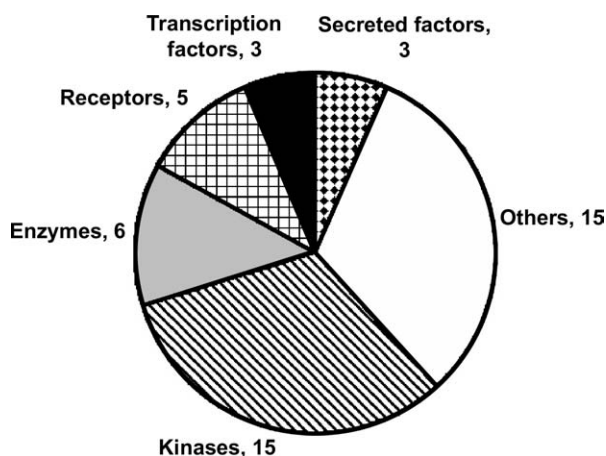

Fig. 5. Distribution of hits. The chart shows hit distribution in various protein classes. The hits consist of 15 kinases, 6 enzymes, 3 extracellular (EC) factors, 5 receptors, 3 transcription factors (TFs) and 15 others.

matrix degradation we measured MMP-13 and Agg-1. These proteinases are produced in high levels by the chondrocytes in OA cartilage and degrade collagen II and aggrecan, two major structural components of cartilage[10]. COX-2 and iNOS were measured as markers of inflammation[33]. The expression of both of these genes can be induced by proinflammatory cytokines and other stimuli. Prostaglandins are important in the generation of joint pain and inflammation and COX-2 inhibitors are most commonly prescribed for OA[2]. Chondrocytes are among a small number of cell types that express high levels of iNOS in response to a single extracellular stimulus such as a cytokine or a microbial product[34]. Abnormal chondrocyte differentiation patterns in OA cartilage include the expression of collagen X and collagen IIA[8,9]. Chondrocyte cloning or cluster formation is a histological hallmark of OA cartilage[21]. This occurs predominantly in areas of cartilage fibrillation suggesting a potential linkage between cluster formation, cell death and cartilage calcification[35]. While alternative or additional markers can be tested, the selected markers are representative of major pathways in OA pathogenesis. The total number of six markers is relatively large for a high-throughput screen.

QPCR was applied to measure induction of these OA phenotypic markers. The advantage of this approach was in the ability to measure the selected six markers for inflammation, matrix degradation and differentiation in the same RNA sample reflecting identical chondrocyte activation status. Chondrocyte cluster formation was measured in three-dimensional agarose cultures. Retrovirally transduced cells were cultured for up to 4 weeks to allow for the formation of large clusters that could be easily identified and isolated.

The results indicate that the functional genomic screens that were developed can identify novel inducers of the OA phenotype. Genes such as FYN oncogene, nuclear
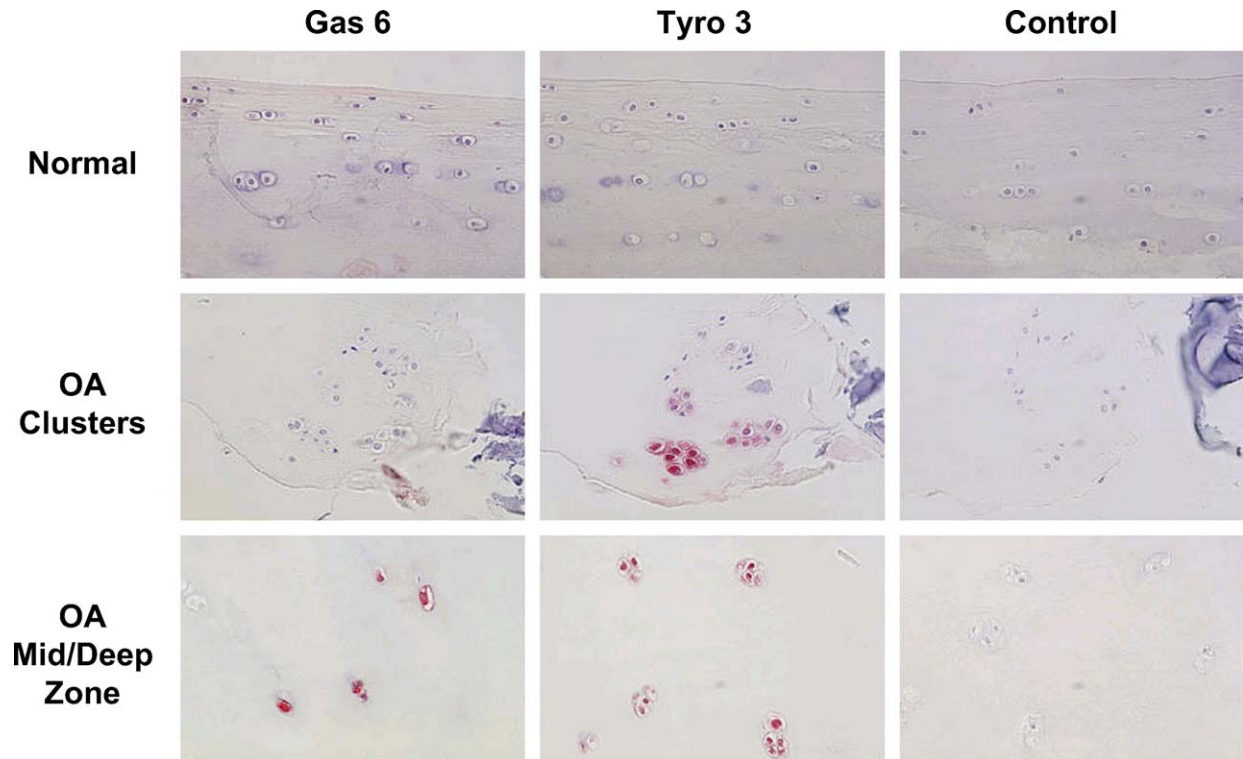
Fig. 6. Increased expression of Tyro-3 and Gas-6 in OA cartilage. The figure shows immunohistochemistry of normal and OA human knee cartilage. Note increased staining in OA clusters and in the mid/deep zone. Control represents staining with an isotype-matched control antibody. Cartilage from at least seven different donors was used for this study and a representative example is shown.

respiratory factor 1, ADP-ribosylation factor 6, Rho-specific guanine nucleotide exchange factor p114 choline kinase like, myeloid/lymphoid leukemia 3 and branched amino transferase 1 have previously not been associated with activation of chondrocytes. Other genes have been previously been shown to regulate chondrocyte function, but these screening assays identified new functions, such as the induction of cluster formation by IL-17 receptor, COMP and VEGF. We show for the first time that genes such as COMP, calcium calmodulin dependent protein kinase-1, casein kinase-2, and Rho-specific guanine nucleotide exchange factor p114 are associated with matrix degradation and chondrocyte proliferation. The two tyrosine kinases Tyro-3 and Axl were the most potent inducers of four OA markers. Their ligand Gas-6 had previously been shown to exert mitogenic effects on chondrocytes[36]. The present findings suggest a new role of this ligand receptor system in chondrocytes. With regard to signaling pathways that can induce OA phenotypic markers, several genes in the PI3-kinase pathway, including PI3K-gamma, PI3K-alpha and GSK3 stimulated Agg-1 and MMP-13 expression. In contrast, RIPK2, a component of the TNFR pathway activated four markers (Agg-1, MMP-13, COX-2 and iNOS), whereas RAR activated only one marker (iNOS). A large number of genes including three novel genes (hypothetical protein FLJ14103, a novel gene abundant in late OA and ECRG4) coordinately induced four markers (Agg-1, MMP-13, COX-2 and iNOS), suggesting that these OA markers are linked to several different signaling pathways.

The screens not only identified novel inducers of chondrocyte activation but also provided the basis for the discovery of novel activation mechanisms. The endonuclease MUS81

was among the most potent inducers of MMP-13, Agg-1, COX-2 and iNOS.

As the primary objective of this study was to develop high-throughput screens in primary HAC, functional analysis was performed only on the basis of cDNA transfer. For a complete and definitive characterization of the function of hits identified in this screen, detailed studies with the corresponding proteins are required. We have shown in the present study that bFGF protein has the same effects as bFGF cDNA transfer. A separate study based on hits identified in this screen was performed for the PI3-kinase pathway and confirms the results obtained in the cDNA transfer experiments[37].

For some of the genes identified in this screen, such as MUS81 endonuclease or ribosomal protein S6 kinase, mechanisms by which they induce gene expression are not obvious. Studies to elucidate these mechanisms have the potential to discover novel functions and pathways. Examples for proteins that have unexpected functions include the tRNA synthase that can regulate intracellular signaling pathways through some of its proteolytic fragments[38].

Although we have identified genes expected to induce expression of OA markers, there are also genes that were expected but did not affect the parameters analyzed. Potential explanations include lack of a proper full-length clone or toxicity associated with high-level gene expression.

A role for the hits identified in the screening assays in OA pathogenesis would require that the genes are expressed in articular cartilage. Data in Tables I and II show that all of the genes that were represented on the DNA array used for these studies are expressed in human articular cartilage.

Increased expression of a gene in OA as compared to normal cartilage may further support its role in the disease process. Several of the genes we identified were previously shown to be increased in OA. These include COMP[39], TNFR[40] and VEGF[41]. Increased expression in cartilage, however, is not essential for a gene to be involved in OA because post-translational changes such as phosphorylation of signaling proteins can determine activation and function.

In conclusion, we have combined genomics and high-throughput screening methods in HAC to identify novel mediators of the OA phenotype. These genes are likely to play key roles in cartilage degradation, inflammation and inappropriate remodeling and could therefore be exploited as potential therapeutic targets for OA.

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